Using electrochemical methods to study the kinetics of laccase-catalyzed oxidation of phenols

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ABSTRACT

In the present work the laccase kinetics upon the oxidation process of phenolic compounds was studied using cyclic voltammetry (CV). Several hydroxybenzoic and cinnamic acid compounds were analyzed and the oxidation kinetics were correlated to their chemical structures. Significant differences were observed, and several structural parameters were identified as enhancers of the reaction rate. Folin-Ciocalteau, UV-Vis Spectrophotometry and Thin Layer Chromatography (TLC) methods were also used to assess the laccase-oxidation process. The TLC results correlated well with those from the CV, while the UV-Vis results do so with those from the Folin-Ciocalteau analysis. Results suggest that intermediate adducts, non-detectable by CV, Folin-Ciocalteau, UV-Vis, and TLC analyses, may be formed during the oxidation process. A new approach for using bare glassy carbon electrodes to detect phenolic compounds and monitor their oxidation using laccase enzymes is presented.

KEYWORDS: Laccase, phenolic compounds, cyclic voltammetry, Folin-Ciocalteau

1. INTRODUCTION

Enzymatic oxidation of phenols for biotechnological applications is an area of increasing interest. Accordingly, the use of oxidative enzymes in bioremediation[1,2], lignocellulose processing[3], organic synthesis[4], medical diagnosis, or pharmaceutical industry are but a few of the potential applications of enzymes. The achievement of efficient enzyme-catalyzed processes is of paramount importance regarding industrial application of laccases. In this regard, the efforts of researchers are usually focused on attaining the optimal reaction conditions and modeling the enzyme kinetics by means of the Michaelis–Menten approach. However, several authors have reported the drawbacks underlying in this model[5,6]. Either using the model or not, laccase kinetics are determined by monitoring one of the parameters involved in the enzymatic reaction, i.e. substrate transformation, product formation or oxygen consumption. The most utilized analytical techniques are: UV spectrometric measurements, by measuring the absorbance change at a given wavelength[2]; electrochemical detection systems either potentiometric[7,8], or amperometric[9]; oxygen consumption monitoring, by measuring the dissolved oxygen concentration in the reaction medium[6]; analyze of the phenol content[10]; and in situ FT-IR reaction monitoring[4].

On the one hand, electrochemistry has emerged as a very promising method to detect and quantify phenols, parabens and its derivatives in different media[11,12]. On the other hand, regarding the use of electrochemical detection systems in enzyme processes, there are three main ways to couple electrodes to enzyme reactions which give rise to the so-called 1st, 2nd and 3rd generation of electrochemical biosensors[13]. In such biosensors, heterogeneous or homogenous catalysis can be accomplished depending on the nature of the electrical connection between the electrode and the redox enzyme. Thus, a wide number of electrode configurations involving the enzyme and mediator free in solution, immobilized, co-immobilized and combinations thereof have been applied over the last thirty years.

To date, several works have reported on laccase oxidation of compounds by using cyclic voltammetry[14,15]. However, few works have reported on this technique with the aim of studying the reaction kinetics. In the works by Fernández-Sánchez et al.[16] laccase was immobilized to the electrode, and the dependence of the substrate concentration on the catalytic current was used to estimate the kinetic parameters. Similarly, in the works by Jarosz-Wilkolazka et al.[17] a comparative kinetic study of a series of phenols was performed using laccase-modified electrodes for amperometric detection. In both studies it is assumed that the action of the electrode is not hindered by the presence of the protein layer on its surface, and diffusion phenomena are not discussed. In the works by Klis et al.[7] a different approach consisting in recording voltammetric data with the enzyme acting in the bulk electrolyte and not from the surface of the electrode is considered. In this interesting approach the effect of the protein layer disappears, and the authors used microelectrodes working under spherical diffusion to follow the enzyme reaction.

With all the previous efforts in mind, in the present work we aimed at using voltammetric methods to study the kinetics of the enzyme laccase during the oxidation of phenolic compounds. Since different behaviors were observed, we could elucidate the link between the chemical structure to the enzyme kinetics. To the best of our knowledge, this is the first time that conventional-sized bare glassy carbon electrode, working under linear diffusion, was used for the assessment of an in-situ catalytic enzyme process in bulk solution.

2. MATERIALS & METHODS

2.1. Enzyme and chemicals

Laccase from *Trametes Villosa*, 588 U/mL, was supplied by Novozymes (Denmark). Eleven phenolic compounds were subjected to laccase-assisted treatments (Table 1). All compounds were purchased from Sigma–Aldrich (Spain).

2.2. Cyclic voltammetry

Voltammetric studies were performed using an Autolab PGSTAT128N (Metrohm, The Netherlands) potentiostat/galvanostat controlled by Nova software version 1.10. All experiments were carried out in a thermostatic, 50 mL, three-electrode configuration cell (Metrohm) (Figure 1a). The working electrode was a glassy carbon electrode with a surface diameter of 3 mm (Metrohm, The Netherlands). A platinum electrode and a silver–silver chloride (Ag/AgCl) electrode (Metrohm, The Netherlands) were used as counter and reference electrode, respectively. Potential was scanned from 0 to 900 mV vs. Ag/AgCl at a scan rate of 30 mV/s. For the peak intensity vs. concentration curves, voltammetric responses were recorded in 50 mL aqueous solution, containing 50 mM tartrate buffer pH 4, 40ºC, at concentrations in the range of 0.05-2 mM of the compound to be studied. The disappearance of the pure compound due to enzyme oxidation was followed by recording the peak intensity at pre-established time periods into the 50 mL cell, with an aqueous solution containing 50 mM tartrate buffer pH 4, 40ºC, at a starting concentration of 2 mM of the compound to be studied and 1,2 U/mL of enzyme (Figure 1d). Enzyme was introduced directly into the cell (Figure 1c). Before each measure, either for calibration and enzyme monitoring, the working electrode was carefully cleaned with distilled water, polished with 0,1 μm alumina powder on a wet pad (Figure 1b), and cleaned again with ethanol and distilled water. As reported in previous works, the enzyme activity loss at 40ºC is not significant within the first 4 hours of incubation[18]; in the present study the reaction time never exceeded 2,5h. Also, even if a slight activity loss existed, it would not have affected the comparative study.

2.3. Samples for phenol content, TLC, and UV-Vis analysis

Enzymatic treatment was performed in a 50 mL beaker containing 2 mM of phenolic compound and 1.2 U/mL laccase dispersed in 50 mM sodium tartrate buffer (pH 4). The phenolic compound was kept under agitation for 10 min at 40ºC. Subsequently, the enzyme was added and the aqueous solution was stirred for 1 h. At 5 minute intervals, 2.5 mL aliquots

were withdrawn, placed in a test tube, and immersed in a bath at 80ºC for 5 minutes for enzyme inactivation.

2.4. Phenol content analysis

Phenol content was measured using the Folin-Ciocalteau micro method adapted by Andrew Waterhouse and readily available on the internet (http://waterhouse.ucdavis.edu/faqs/folin-ciocalteau-micro-method-for-total-phenol-in-wine). First, a calibration curve (absorbance vs. concentration) was obtained using several GA solutions. The absorbance was recorded at 765 nm. Three readings were averaged for each sample. Results were reported as mg/L Gallic Acid Equivalents (GAE).

2.5. UV-Vis spectrophotometry

The oxidation of compounds was examined by UV–Vis spectrophotometry, using a Thermo Scientific Evolution 600 instrument. All samples were diluted 10-fold in distilled water prior UV-Vis analysis. The reference polymethyl methacrylate cuvette (1 mL) was filled with 50 mM sodium tartrate buffered at pH4, and 1.2 U/mL laccase. Aliquots from the enzymatic reaction were measured in the range 200-900 nm.

2.6. TLC analysis

For TLC tests, 200 µL of sample were spotted dropwise using a 10 µL pipette on a silica gel precoated plate. Then, the plate was introduced in a glass and developed with mobile system of toluene:ethyl-acetate:formic acid (5:5:1, v/v/v). The plate was eluted twice, allowing it to air-dry between elutions. Finally, plates were sprayed with $FeCl_3$ (2% in ethanol) for visualization. The disappearance of the phenolic compound upon enzyme reaction was identified by comparison with the time 0 solution, after development of the plate.

3. RESULTS & DISCUSSION

3.1. Enzyme reaction monitoring. Role of chemical structure in laccase-oxidation

The relationship between concentration of phenols and the voltammetric response was studied in previous works[12,19]. In the present work, high linearity has been achieved with no modification of the glassy carbon working electrode, but polishing and cleaning it between measurements to avoid fouling effects (Figure 1c). We studied intensity vs. concentration in the range 0.05-2 Mm, and voltammograms for all compounds were obtained (Figure 1). The curves provided a coefficient of determination above 0,9 for all compounds (Table 1). The hydroxybenzoic (HB) and hydroxycinnamic (HC) compounds studied (Table 1) are structurally similar except that HC have an ethylenic chain attached to the aromatic ring. The compound's structure plays a paramount role in the oxidation rate promoted by laccase (Figure 2). Thus, HC compounds seem to oxidize faster than HB. This is evident when comparing sinapic and syringic acids, which have the same phenolic moiety with the difference that the former has HB while the latter HC structure. The same observation can be drawn by comparing sinapyl aldehyde and syringaldehyde, but in this case the compounds are phenolic aldehydes.

Depending on whether the compound is under the form of acid or phenolic aldehyde, the oxidation speed is affected. Hence, by comparing two HB compounds with the same phenolic moiety like syringaldehyde and syringic acids, the oxidation rate is faster in the latter. This is indicative that the laccase oxidation is faster for acid forms. This observation can be confirmed by comparing two HC compounds with the same phenolic structure. This is the case of sinapyl aldehyde and sinapic acids couple, being the oxidation rate faster for sinapic acid. Another interesting observation is on the type of phenol substituents within the compound. For example, comparing syringic and gallic acid compounds (both HB structure), the only difference is that syringic has methoxy goups, while gallic acid has hydroxyl groups in the meta-positions. Then, the oxidation kinetic is faster for syringic acid, indicating an

increased oxidation rate for compounds bearing methoxy substituents. This can also be verified in HC compounds, by comparing ferulic and caffeic acids.

The presence or absence of substituents has also an effect on the reaction speed. By comparing sinapic and ferulic acids structures, the difference is the methoxy groups in both meta positions for sinapic, while ferulic has only one methoxy. Now, in spite that the oxidation is very fast in both compounds, it seems to be faster with ferulic since less oxidable product is available after the first 5 minutes. Comparison between ferulic and p-coumaric acids (the latter with any substituent in the meta positions) is difficult because both compounds undergo very fast oxidations. To corroborate this observation, the reaction speed of syringaldehyde and vanillin (which are the HB counterparts of sinapic and ferulic acids under the form of phenolic aldehydes) can be analyzed. In this case, results confirm that the absence of the methoxy group in vanillin with respect to syringaldehyde causes a faster laccase-assisted oxidation.

Considering all the previous observations it can be stated that there are several aspects regarding the chemical structure of compounds that would determine the oxidation kinetic. To summarize, the following parameters seem to promote and increase the reaction speed: i. compounds with HC structure, ii. presence of methoxy rather than hydroxy substituents in the meta positions, iii. higher speed for acid with respect the aldehyde forms, and vi. higher affinity (or oxidation rate) with the absence of substituents.

3.2 Phenol content determination

Samples of all the studied phenolic compounds were taken at given intervals for different analysis as shown in the Materials and Methods section. Fristly, samples were analyzed for phenol content by means of Folin-Ciocalteu method. The general behavior is to experience a drastic drop in the phenol content of about the 50% upon the first fifth minutes of reaction (Figure 3a). After this first drop, the phenol content stabilizes for all compounds

during the enzymatic reaction. The inset in Figure 3a shows the % decrease during the first 5 minutes. As it is shown, for the main part of compounds the drop is of about 50-70%, except for Syringic Acid, which experiences a drop higher than the 80%. Results show that after the enzymatic reaction a significant amount of phenols is still present. However, taking into account the CV results in the previous section, if such phenolic compounds are there, they may be new non electrode-oxidizable adducts, formed upon enzymatic oxidation. Also, we have to take into account that the Folin method measures the total reducing capacity of the samples and not only phenolic compounds. The reagent has also been shown to be reactive towards various non-phenolic compound classes[20]. Some non-phenolic but Folin-reactive species may have formed, affecting the phenol content measurement by this method.

3.3 UV-Vis and TLC analysis

The aliquot samples from the enzyme reaction at different times were further studied with UV-Vis spectrophotometry and TLC. Regarding UV-Vis analysis all the studied compounds presented absorbance peaks between 200 and 400 nm, so they were not colored to naked eye. After the enzyme oxidation, all compounds still showed such peaks, but also showed absorbance in the whole spectra, being this absorbance more significant for the short wavelengths, i.e 400 to 500 nm. In all compounds, the absorbance curves did not show significant shifts after the first 5 minutes of oxidation, which is in accordance with the phenol content analysis discussed in the previous paragraphs. Figure 3b shows the changes on the spectra corresponding to GA compound as example, although all compounds showed similar behavior. All compounds presented colored appearance after enzyme oxidation. However, the absorbance peak shifted several nanometers from their original position, and also the peak intensity was increased or decreased depending on the compound and the reaction time.

A first TLC analysis revealed that all the studied compounds were movable by the mobile phase and presented marked and clear spots with an Rf value of about 0.6. Then, the

reaction aliquots were spotted in separate plates, taking a single plate per compound. Results revealed that, with the exception of gallic acid, after the first 5 minutes of reaction any amount of the pure compounds was still present. This was indicative that the enzyme reaction caused important changes in the structure of compounds during the first minutes. However, for gallic acid, the spot corresponding to the pure compound could be observed (although weaker) up to the first 40 minutes of reaction (see GA example in Figure 3c). This fast transformation of the pure compounds does not necessarily imply the absence of other electrode-oxidizable species, as demonstrated with CV. Regarding CV, only few compounds suffered a fast 5-10 minutes' oxidation (FA, pCA, SPA and TYR). For the rest of compounds, similar voltammograms with respect to the one of the pure compound were obtained after the first 5 minutes of reaction. This is an indication that in spite the enzyme causes fast structural changes in the studied compounds so they are not viewable by TLC, the remaining molecule may be similar to its original. Thus, similar voltammograms (only slight peak shift, and obvious decrease in intensity) were obtained after the first 5 minutes. It is possible that CV is not accurate enough to distinguish between the pure and the initial forms of the oxidized molecules. It is not until oxidation reaches decayed/depleted species that the peak intensity decreases. In the TLC plates corresponding to Syringic acid and Syringaldehyde compounds (see SA example in Figure 3d) no spot corresponding to the pure compound was observed upon reaction. However, new spots at different positions (different Rf) were observed at naked eye and without spaying the plate. This is an evidence of the formation of new adducts during the laccase reaction. For the rest of the compounds, the spots corresponding to the different time periods seem not to have moved, indicating that the new adducts may be no longer movable by this mobile phase.

4. CONCLUSIONS

The present work demonstrated that high-linearity intensity vs. concentration calibration curves (>0,9 R^2), can be obtained for phenolic compounds using cyclic voltammetry and a glassy carbon electrode. The laccase-oxidation process of phenolic compounds can also be successfully monitored with CV, and is possible to use the resulting concentration vs. time curves to link the behavior to the chemical structure. As demonstrated after conducting Folin-Ciocalteau, UV-Vis spectrophotometry and TLC analysis as alternative methods for the characterization of the laccase reaction, the voltammetric method may be more accurate since it allows the detection of intermediate reaction products.

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REFERENCES

(1) Kandelbauer, A.; Maute, O.; Kessler, R. W.; Erlacher, A.; Gübitz, G. M. Study of Dye Decolorization in an Immobilized Laccase Enzyme-Reactor Using Online Spectroscopy. *Biotechnol. Bioeng.* **2004**, *87* (4), 552.

(2) Sóti, V.; Jacquet, N.; Apers, S.; Richel, A.; Lenaerts, S.; Cornet, I. Monitoring the Laccase Reaction of Vanillin and Poplar Hydrolysate. *J. Chem. Technol. Biotechnol.* **2016**, *91* (6).

(3) Fillat, A.; Roncero, M. B.; Vidal, T. Assessing the Use of Xylanase and Laccases in Biobleaching Stages of a TCF Sequence for Flax Pulp. *J. Chem. Technol. Biotechnol.* **2011**, *86* (12), 1501.

(4) Engelmann, C.; Illner, S.; Kragl, U. Laccase Initiated C-C Couplings: Various

Techniques for Reaction Monitoring. *Process Biochem.* **2015**, *50* (10).

(5) Ritchie, R. J.; Prvan, T. Current Statistical Methods for Estimating the Km and Vmax of Michaelis-Menten Kinetics. *Biochem. Educ.* **1996**, *24* (4).

(6) Kurniawati, S.; Nicell, J. A. Kinetic Model of Laccase-Catalyzed Oxidation of Aqueous Phenol. *Biotechnol. Bioeng.* **2005**, *91* (1).

(7) Klis, M.; Rogalski, J.; Bilewicz, R. Voltammetric Determination of Catalytic Reaction Parameters of Laccase Based on Electrooxidation of Hydroquinone and ABTS. *Bioelectrochemistry* **2007**, *71* (1).

(8) Palmore, G. T. R.; Kim, H.-H. Electro-Enzymatic Reduction of Dioxygen to Water in the Cathode Compartment of a Biofuel Cell. *J. Electroanal. Chem.* **1999**, *464* (1).

(9) Zille, A.; Munteanu, F.-D.; Gübitz, G. M.; Cavaco-Paulo, A. Laccase Kinetics of Degradation and Coupling Reactions. *J. Mol. Catal. B Enzym.* **2005**, *33* (1–2), 23.

(10) Alper, N.; Acar, J. Removal of Phenolic Compounds in Pomegranate Juices Using Ultrafiltration and Laccase-Ultrafiltration Combinations. *Nahrung - Food* **2004**, *48* (3), 184.

(11) Nissim, R.; Compton, R. G. Introducing Absorptive Stripping Voltammetry: Wide Concentration Range Voltammetric Phenol Detection. *Analyst* **2014**, *139* (22), 5911.

(12) Spătaru, T.; Spătaru, N. Voltammetric Detection of Phenol at Platinum-Polytyramine Composite Electrodes in Acidic Media. *J. Hazard. Mater.* **2010**, *180* (1–3), 777.

(13) Das, P.; Das, M.; Chinnadayyala, S. R.; Singha, I. M.; Goswami, P. Recent Advances on Developing 3rd Generation Enzyme Electrode for Biosensor Applications. *Biosens. Bioelectron.* **2016**, *79*, 386.

(14) Aracri, E.; Tzanov, T.; Vidal, T. Use of Cyclic Voltammetry as an Effective Tool for Selecting Efficient Enhancers for Oxidative Bioprocesses: Importance of pH. *Ind. Eng. Chem. Res.* **2013**, *52* (4), 1455.

(15) Díaz-González, M.; Vidal, T.; Tzanov, T. Phenolic Compounds as Enhancers in Enzymatic and Electrochemical Oxidation of Veratryl Alcohol and Lignins. *Appl. Microbiol. Biotechnol.* **2011**, *89* (6), 1693.

(16) Fernández-Sánchez, C.; Tzanov, T.; Gübitz, G. M.; Cavaco-Paulo, A. Voltammetric Monitoring of Laccase-Catalysed Mediated Reactions. *Bioelectrochemistry* **2002**, *58* (2).

(17) Jarosz-Wilkołazka, A.; Ruzgas, T.; Gorton, L. Amperometric Detection of Mono- and Diphenols at Cerrena Unicolor Laccase-Modified Graphite Electrode: Correlation between Sensitivity and Substrate Structure. *Talanta* **2005**, *66* (5), 1219.

(18) Ibarra, D.; Romero, J.; Martínez, M. J.; Martínez, A. T.; Camarero, S. Exploring the Enzymatic Parameters for Optimal Delignification of Eucalypt Pulp by Laccase-Mediator. *Enzyme Microb. Technol.* **2006**, *39* (6), 1319.

(19) Radovan, C.; Cinghiţă, D.; Manea, F.; Mincea, M.; Cofan, C.; Ostafe, V. Electrochemical Sensing and Assessment of Parabens in Hydro-Alcoholic Solutions and Water Using a Boron-Doped Diamond Electrode. *Sensors* **2008**, *8* (7), 4330.

(20) Everette, J. D.; Bryant, Q. M.; Green, A. M.; Abbey, Y. A.; Wangila, G. W.; Walker, R. B. Thorough Study of Reactivity of Various Compound Classes toward the Folin-Ciocalteu Reagent. *J. Agric. Food Chem.* **2010**, *58* (14), 8139.

LIST OF FIGURE AND TABLE CAPTIONS

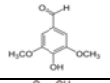
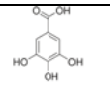
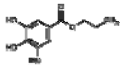
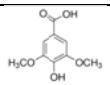
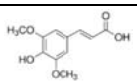
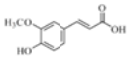
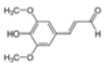
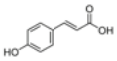
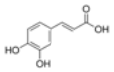
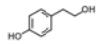
Figure 1. Schematic of the enzymatic reaction conditions in the electrochemical cell (a), electrode polishing (b), enzyme introduction in the cell (c), and example of the voltammetric response evolution upon laccase oxidation for GA compound (d).

Figure 2. Concentration vs. time curves upon enzymatic reaction obtained by monitoring the anodic peak. Voltammograms were obtained at 30 mV/s, pH 4, 40ºC between 0-900 mV. Inset: Compounds arranged in decreasing oxidation time.

Figure 3. Concentration vs. reaction time for a 2 mM starting solution upon enzymatic reaction measures with the Folin-Ciocalteau micro method (a), GA UV-Vis curves of aliquots taken at 5 minutes' intervals from the enzymatic reaction (2 mM GA, 40ºC, pH 4) within the range 200-900 nm (b), and TLC analysis corresponding to GA (c) and SA (d) compounds as examples.

Table 1. Chemical structure, and parameters of the intensity vs. concentration curves of the studied compounds in the range 0.05-2 mM. Voltammograms obtained at 30 mV/s, pH 4, 40ºC between 0-900 mV.

Table 1

		Structure	Molecular weight	Slope [µA/mM]	y intercept [µA]	R^2
HYDROXYBENZOIC	**Syringaldehyde (SA)**		*182.17*	*12.821*	*1.3779*	*0.9987*
	Gallic acid (GA)		*170.12*	*25.662*	*4.3957*	*0.9966*
	Propyl Gallate (PG)		*212.2*	*17.297*	*5.6774*	*0.9190*
	Syringic acid (SYR)		*198.18*	*25.617*	*1.9689*	*0.9987*
	Vanillin (V)		*152.15*	*10.711*	*3.9899*	*0.9473*
HYDROXYCINNAMIC	**Sinapic acid (SPA)**		*224.21*	*5.8795*	*2.6231*	*0.9371*
	Ferulic Acid (FA)		*149.19*	*6.1319*	*3.8129*	*0.8632*
	Sinapyl aldehyde (SPAL)		*208.21*	*8.2888*	*1.6133*	*0.9911*
	p-Coumaric acid (pCA)		*164.16*	*4.5781*	*3.8581*	*0.8494*
	Caffeic Acid (CA)		*180.16*	*21.907*	*1.5086*	*0.9975*
	Tyrosol (TYR)		*138.164*	*5.634*	*3.8963*	*0.8667*

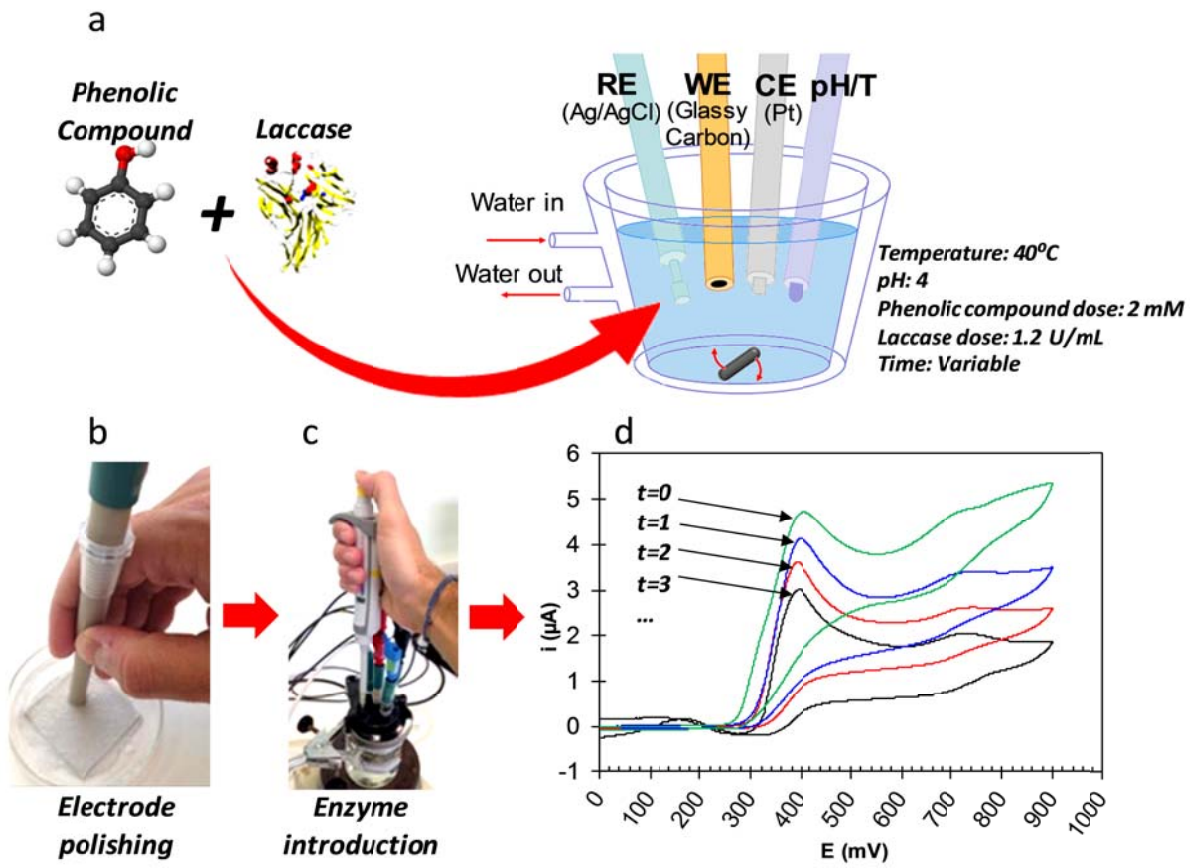

a
Phenolic Compound
Laccase
RE (Ag/AgCl)
WE (Glassy Carbon)
CE (Pt)
pH/T
Water in
Water out
Temperature: 40°C
pH: 4
Phenolic compound dose: 2 mM
Laccase dose: 1.2 U/mL
Time: Variable
b
c
d
Electrode polishing
Enzyme introduction
t=0
t=1
t=2
t=3
...
i (µA)
E (mV)

Figure 1

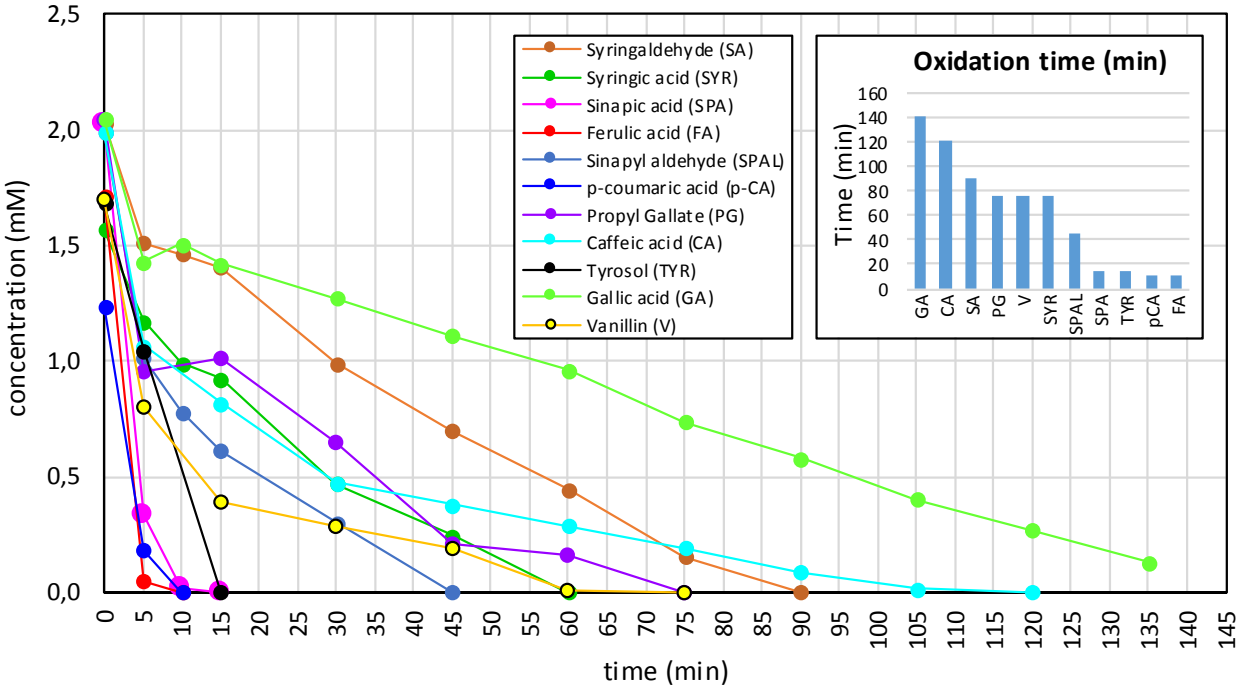

concentration (mM)
time (min)
Syringaldehyde (SA)
Syringic acid (SYR)
Sinapic acid (SPA)
Ferulic acid (FA)
Sinapyl aldehyde (SPAL)
p-coumaric acid (p-CA)
Propyl Gallate (PG)
Caffeic acid (CA)
Tyrosol (TYR)
Gallic acid (GA)
Vanillin (V)
Oxidation time (min)
Time (min)
GA
CA
SA
PG
V
SYR
SPAL
SPA
TYR
pCA
FA

Figure 2

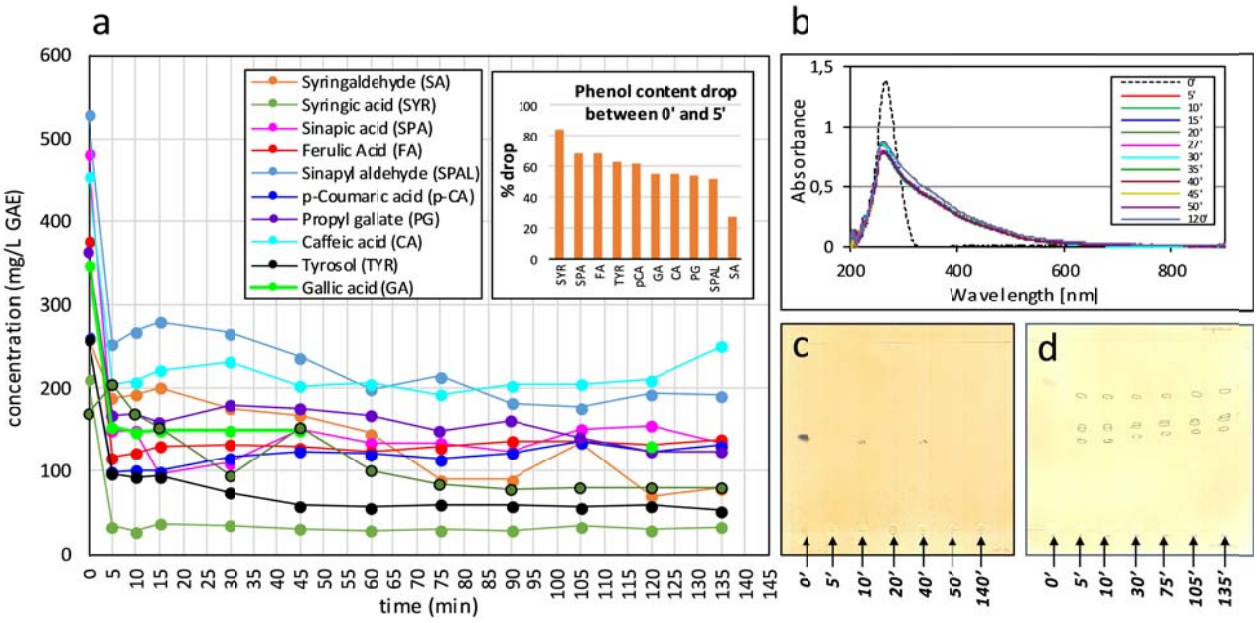

a
b
c
d
concentration (mg/L GAE)
time (min)
Syringaldehyde (SA)
Syringic acid (SYR)
Sinapic acid (SPA)
Ferulic Acid (FA)
Sinapyl aldehyde (SPAL)
p-Coumaric acid (p-CA)
Propyl gallate (PG)
Caffeic acid (CA)
Tyrosol (TYR)
Gallic acid (GA)
Phenol content drop between 0' and 5'
% drop
SYR
SPA
FA
TYR
pCA
GA
CA
PG
SPAL
SA
Absorbance
Wavelength [nm]
0'
5'
10'
20'
40'
50'
140'
0'
5'
10'
30'
75'
105'
135'

Figure 3

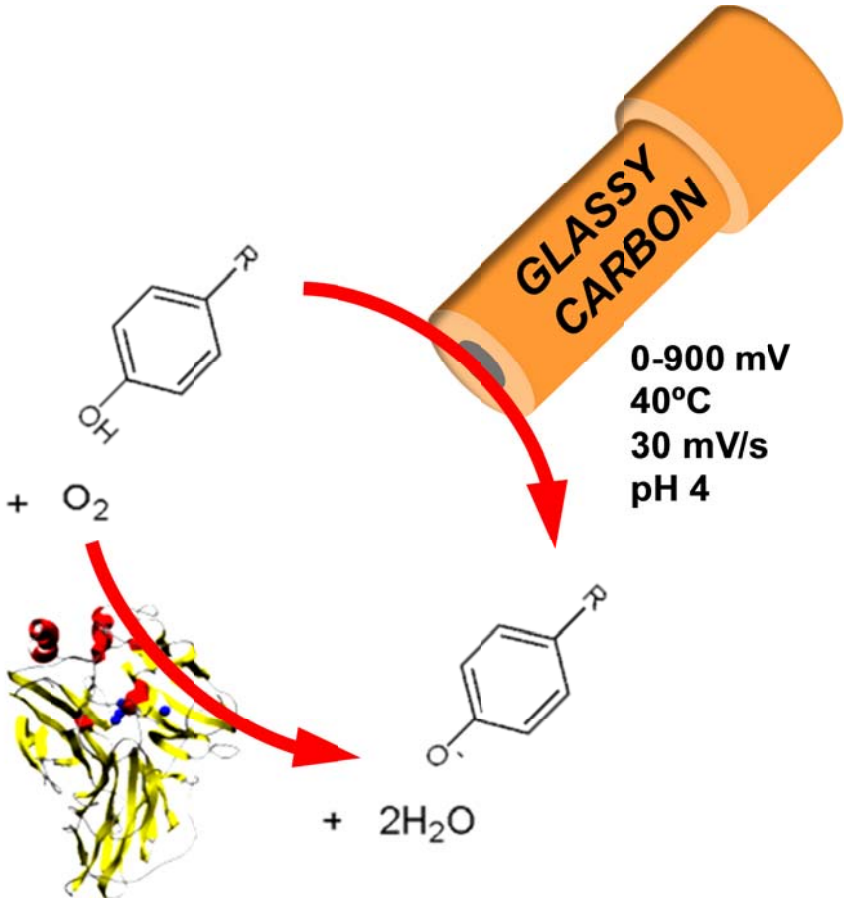


Table of Contents (TOC) /Abstract graphic